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ORIGINAL ARTICLE

Anticancer Potential of Nutraceutical Formulations in MNU-induced Mammary Cancer in Sprague Dawley Rats

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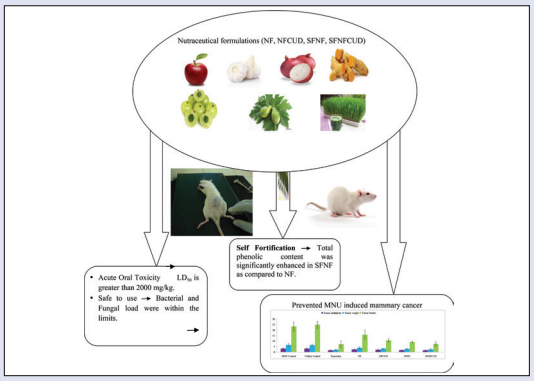
ABSTRACT

Background: Nutraceuticals help in combating some of the major health problems of the century including cancer, and 'nutraceutical formulations' have led to the new era of medicine and health. **Objective:** To develop different nutraceutical formulations and to assess the anticancer potential of nutraceutical formulations in N-methyl-N-nitrosourea (MNU)-induced mammary cancer in Sprague Dawley rats. **Materials and Methods:** Different nutraceutical formulations were prepared using fine powders of amla, apple, garlic, onion, papaya, turmeric, and wheat grass with and without cow urine distillate. Total phenolic content, acute oral toxicity, and microbial load of nutraceutical formulations were assessed. The anticancer potential of nutraceutical formulations was evaluated against MNU-induced mammary cancer in female Sprague Dawley rats. **Results:** Improvement in total phenolic content was significant ($P < 0.001$) after self-fortification process. Toxicity studies showed that the nutraceutical formulations were safe to use in animals. Microbial load was within the limits. Significant longer tumor-free days ($P < 0.01$), lower tumor incidence ($P < 0.01$), lower tumor multiplicity ($P < 0.05$) and tumor burden ($P < 0.01$) were observed for nutraceutical formulation-treated groups. **Conclusion:** Combination of whole food-based nutraceuticals acted synergistically in the prevention of mammary cancer. Further, the process of fortification is novel and enhanced the anticancer potential of nutraceutical formulations.

Key words: Nutraceuticals, Mammary Cancer, Fortification, Cow urine distillate

SUMMARY

Nutraceuticals help in combating some of the major health problems of the century including cancer, and 'nutraceutical formulations' have led to the new era of medicine and health. In this study, different nutraceutical formulations using fine powders of amla, apple, garlic, onion, papaya, turmeric, and wheat grass with and without cow urine distillate. Total phenolic content, acute oral toxicity, and microbial load of nutraceutical formulations were assessed. The anticancer potential of nutraceutical formulations was evaluated against MNU-induced mammary cancer in female Sprague Dawley rats. Improvement in total phenolic content was observed after self-fortification process. Toxicity studies showed that the nutraceutical formulations were safe to use in animals. Microbial load was within the limits. Longer tumor-free days, lower tumor incidence, lower tumor multiplicity and tumor burden were observed for nutraceutical formulation-treated groups. This suggests that combination of whole food-based nutraceuticals acted synergistically in the prevention of mammary cancer. Further, the process of fortification enhanced the anticancer potential of nutraceutical formulations.



Abbreviations used: HMNU: N-methyl-N-nitrosourea, CAM: Complementary and Alternative Medicine, NF: Nutraceutical Formulation, SFNF: Self-Fortitfied Nutraceutical Formulation, NFCUD: Nutraceutical Formulation fortified with Cow Urine Disstillate, SFNFCUD: Self-Fortified Nutraceutical Formulation fortified with Cow Urine Disstillate, CPCSEA: Committee for the Purpose of Control and Supervision of Experiments on Animals, OECD: Organisation for Economic Co-operation and Development, TPC: Total Phenolic Content, ANOVA: Analysis of Variance, GAE: Gallic Acid Equivalent, cfu/g: Colony forming unit per g

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INTRODUCTION

Although the incidence of many types of cancer has declined in the last few decades, the prevalence of breast cancer has been rising worldwide, possibly due to demographic and environmental factors and improvements in diagnosis and is one of the most important public health problems due to its growing incidence and mortality rates.[1,2] All over the world, breast cancer is commonest or second commonest cancer in women and accounts for 23% of all newly occurring cancers in women and represents 13.7% of all cancer deaths. The global burden of breast cancer doubled between 1975 and 2000. It seems certain to double again by 2030 and the great majority of this burden will fall on low-income and lower middle-income countries like India, where the resources to deal with the current situation are absent to a great degree.[3]

In recent years, there has been an explosion of life-saving treatment advances against breast cancer, bringing new hope and excitement. Instead of only one or two options, today there is an overwhelming menu

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of treatment choices that fight the complex mix of cells in each individual cancer. The treatment choices are surgery, radiation therapy, hormonal (antiestrogen) therapy, and/or chemotherapy. However, these treatment strategies were not showing satisfying results and even causing many side effects.[4] Hence, researchers are working to better understand the value and benefit of complementary medicine in breast cancer treatment.

The new era of 21st century showed enormous growing awareness of nutraceuticals as potent therapeutic supplements with accepted concept of nutraceutical medicine as a new branch of complementary and alternative medicine (CAM). Further, the healthcare industry also demonstrated the shift of growing population from medical treatment of cancer toward nonprescription nutraceuticals as self-medication in cancer management and prevention. Hence, this study was aimed to develop nutraceutical formulations using amla fruit, apple fruit, garlic and onion bulbs, papaya leaves, turmeric rhizomes, and wheat grass and to evaluate anticancer potential in MNU-induced mammary cancer in Sprague Dawley rats.[5]

MATERIAL AND METHODS

Chemicals and reagents

MNU and Folin–Ciocalteu reagent were purchased from Sigma Chemical Co. (St. Louis, MO); Tamoxifen tablets (Cytotam, Cipla Ltd.) were collected from Mahatma Gandhi Cancer Hospital, Visakhapatnam. Cow urine distillate was collected from Iskon temple, Visakhapatnam. All other chemicals and reagents were obtained from commercial sources and were of analytical grade.

Nutraceuticals

All the nutraceuticals used in the preparation of formulations were of fine grade and were collected from the local market.

Animals

Virgin, female Swiss albino mice, weighing 25–32 g and female Sprague Dawley rats of 35 days of age were obtained from Teena Labs. Pvt. Ltd., Hyderabad, Andhra Pradesh, India. The animal house was well ventilated. The animals were housed in large spacious hygienic cages during the course of the experimental period. The animals were housed under standard laboratory conditions of temperature (21 ± 1°C); relative humidity 50 ± 15% with a 12-h light/dark schedule. They were provided with food (Nutrimix Std-1020, Nutrivet Laboraotories, Pune.) and water *ad libitum*.

The procedures followed in this study were in accordance with the guidelines of the Institutional Animal Ethics Committee (Regd. No. 516/01/a/CPCSEA) on the Use and Care of Animals.

Preparation of carcinogen

MNU was prepared according to the method described by Thompson and Adlakha in 1991.[6] MNU was dissolved immediately before use in 0.9% NaCl and adjusted with 0.05% acetic acid to pH value 4. Fresh solutions were prepared before application.

Preparation of nutraceutical formulations

Four types of nutraceutical formulations are prepared. Plain nutraceutical formulation (NF) was prepared by using fine dried powders of apple fruit, amla fruit, garlic bulbs, onion bulbs, papaya leaves, turmeric rhizomes, and wheat grass in equal quantity. Self-fortitfied nutraceutical formulation (SFNF) was prepared by using fine powders of self-fortified amla, self-fortified papaya, self-fortified wheat grass along with apple, garlic, onion, and turmeric powders. Self-fortification was done by deliberately fortifying the powder with their respective freshly prepared juice (100 g of powder fortified with 50 mL of juice) for three times before adding to the final formulation. Similarly, NFCUD and SFNFCUD were prepared by fortifying NF and SFNF with cow urine distillate (100 g of formulation fortified with 50 mL of cow urine distillate each time).

Total phenolic content

The total phenolic content (TPC) was determined by Folin–Ciocalteu assay using tannic acid as standard. One hundred microliter of sample containing 0.2 mg of nutraceutical formulation was dispensed into a test tube, 100 µl of distilled water and 2.5 mL of Folin–Ciocalteu reagent was added respectively and shaken thoroughly; after 3 min, 2.0 mL of 7.5% sodium carbonate solution was added and the mixture was incubated at 45°C in a water bath for 40 minutes. Absorbance was measured at 760 nm against a blank. The blank is a mixture of 0.2 mL of distilled water, 2.5 mL of Folin–Ciocalteu reagent and 2.0 mL of 75% sodium carbonate. The total phenolic content was expressed as tannic acid equivalent (mg of TAE/g sample) through the calibration curve of tannic acid. All tests were carried out in triplicate.[7]

Acute oral toxicity

Acute oral toxicity test was performed as per Organization for Economic Co-operation and Development (OECD) guidelines 420. In brief, all the nutraceutical formulations were administered orally to different groups of mice at a dose level of 2000 mg/kg body weight. These animals were observed for 24 hours, and then for 14 days.[8]

Microbial load

Each nutraceutical sample (10 g) was suspended in 100 mL lactose broth separately and mixed thoroughly. The sample solution of 10 mL of was diluted to 100 mL with sterilized NaCl- Peptone solution. This was used as crude sample. From this crude stock, serial dilutions were made using sterilized NaCl-Peptone solution. In sterilized conditions, 1 mL of crude stock and dilutions were inoculated to 20 mL of sterilized bacterial medium and fungal medium respectively and poured in petri plates and kept for solidification. After solidification, bacterial plates were incubated at 37°C and fungal plates were in incubated at 25°C. Finally colonies were counted.[9]

Evaluation of mammary gland carcinogenesis

At the age of 43 days, female Sprague Dawley rats were randomly divided into six groups consisting of eight animals in each group: Group I: Rats were induced with mammary carcinoma using N-Methyl N-nitrosourea (50 mg/kg body weight, i.p.). Group II: Mammary carcinoma was induced (as in Group I) and treated with Standard drug Tamoxifen (2 mg/kg body weight, p.o.). Group III: Mammary carcinoma was induced (as in Group I) and treated with plain nutraceutical formulation (NF; 500 mg/kg body weight, p.o.). Group IV: Mammary carcinoma was induced (as in Group I) and treated with self-fortified nutraceutical formulation (SFNF; 500 mg/kg body weight, p.o.). Group V: Mammary carcinoma was induced (as in Group I) and treated with plain nutraceutical formulation fortified with cow urine distillate (NFCUD; 500 mg/kg body weight, p.o.). Group VI: Mammary carcinoma was induced (as in Group I) and treated with self-fortified nutraceutical formulation fortified with cow urine disstillate (SFNFCUD; 500 mg/kg body weight, p.o.). All the treatments were started from one week before MNU administration once in a day for twenty four weeks.

Rats were weighed and palpated for tumors every week from 4th week until 24th week after MNU administration. A tumor was defined as a discrete palpable mass recorded on at least 2 consecutive weeks. Tumor incidence for each treatment was calculated as the percentage of animals

with one or more palpable tumors. Tumor multiplicity was calculated as the average number of tumors per animal in each treatment group. Mean latency of tumor onset for each treatment group was calculated as the mean time interval (in weeks) from MNU injection to the appearance of the first palpable tumor. Dimensions (length × width) of the tumors were measured using a digital caliper, and the tumor burden was calculated using the formula: 0.5 × length × width.[10] At the end of the experiment, rats from each experimental group were sacrificed by decapitation, and mammary tumors were excised for further analysis.

Histopathology

Formalin-fixed tissues were processed routinely through graded ethanol, xylene, and paraffin embedding to obtain 5 µm thick sections and stained with haematoxylin and eosin (H and E) stain for histopathological examination.

Statistical analysis

All the values were represented as mean ± SEM and analyzed by one-way analysis of variance (ANOVA) followed by Tukeys post hoc test using graph pad prism version 5.0. The results were considered statistically significant when the $P \leq 0.05$.

RESULTS

Total phenolic content, acute oral toxicity and microbial load

From the results, it was observed that total phenolic content was significantly enhanced in SFNF as compared to NF [Table 1]. From acute toxicity studies, we found that there were no toxic symptoms at a dose level of 2000 mg/kg. We also found that both bacterial load and fungal load were within the limits [Table 2].

Table 1: Total phenolic content of NF and SFNF (mg GAE /100 g)

Formulation	Total phenolic content (mg GAE/100 g)
NF	88.31 ± 3.55
SFNF	152.09 ± 4.81***

All the values are expressed as the mean ± SEM. *** $P < 0.001$ compared to the NF.

Table 2: Microbial load of nutraceutical formulations

S. No.	Formulation	Bacterial load (Aerobic) (cfu/g of sample)	Fungal load (cfu/g of sample)
1.	NF	3.0×10^4	810
2.	NFCUD	3.4×10^4	795
3.	SFNF	6.1×10^4	870
4.	SFNFCUD	7.7×10^4	910

cfu/g: Colony forming unit per g of sample

Evaluation of mammary gland carcinogenesis

Preventive treatment of SFNF, NFCUD, and SFNFCUD significantly delayed tumor latency as compared to MNU control, whereas NF treatment showed insignificant effect on tumor latency. Similarly, tumor incidence and tumor multiplicity were also significantly reduced in NFCUD, SFNF, and SFNFCUD treated rats as compared to MNU control rats but not in NF treated rats. But, all the four formulations; NF, NFCUD, SFNF, and SFNFCUD significantly reduced tumor weight and tumor burden as compared to MNU control rats showed in Figures 1, 2, 3, and 4 and results were tabulated in Table 3.

Histopathology

The majority of the tumors that developed in the MNU control group rats were adenocarcinomas with few adenomas and fibroadenomas. Carcinomas retained normal architecture of the gland and invaded surrounding tissues. The tissue invasion was mostly local. Massive stromal response demonstrated by inflammatory infiltration and fibrosis was frequently observed. Necrosis was often observed in few invasive carcinomas. In contrast, the tumors that developed in the nutraceutical formulation supplemented rats were mix of fibroadenomas and adenomas. The lesions were moderately cellular and exhibited papillary structure. The stromal response and vascular proliferation was much less than that seen in the adenocarcinoma of MNU control rats showed in Figures 5 and 6.

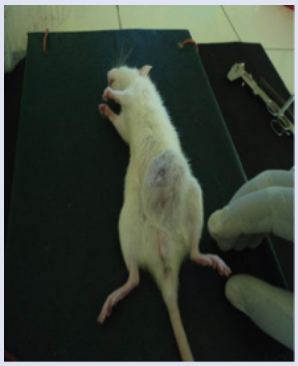

Figure 1: Intact large tumor of MNU control rat.

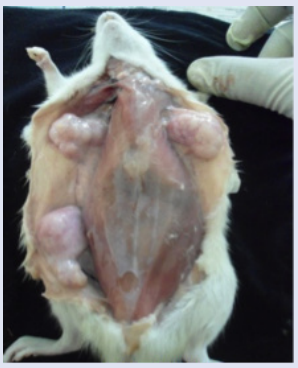

Figure 2: Intact multiple tumors of the MNU control rat.

Table 3: Preventive effect of different types of nutraceutical formulations in MNU induced mammary cancer

Group	Tumor latency (weeks)	Tumor incidence (%)	Tumor multiplicity/rat	Tumor weight/rat (g)	Tumor burden/rat (cm^2)
MNU Control	14.57 ± 0.37	6/7 (85.7)	3.14 ± 0.67	6.50 ± 1.17	23.4 ± 4.01
Tamoxifen (2 mg/kg)	20.50 ± 0.50***	2/8 (25)***	1. 5 ± 0.50**	2.01 ± 0.50**	7.21 ± 3.20***
NF (500 mg/kg)	16.25 ± 0.25	5/8 (62.5)	2.40 ± 0.50	3.87 ± 1.14*	15.78 ± 4.33**
NFCUD (500 mg/kg)	18.25 ± 0.47**	3/8 (37.5)**	2.01 ± 0.57*	3.13 ± 0.37*	10.74 ± 1.57***
SFNF (500 mg/kg)	19.00**	3/8 (37.5)**	1.67 ± 0.33*	2.85 ± 0.64*	9.17 ± 0.67***
SFNFCUD (500 mg/kg)	19.50 ± 0.50**	2/8 (25)***	1.5 ± 0.50**	2.30 ± 0.90**	7.41 ± 1.78***

All the values were expressed as mean ± SEM., *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared to the MNU Control ($n = 8$).

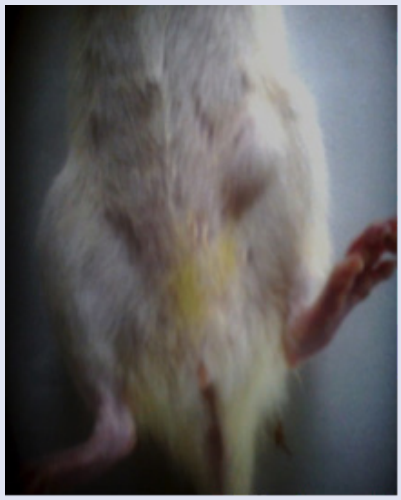

Figure 3: Intact small tumor of SFNF treated rat.

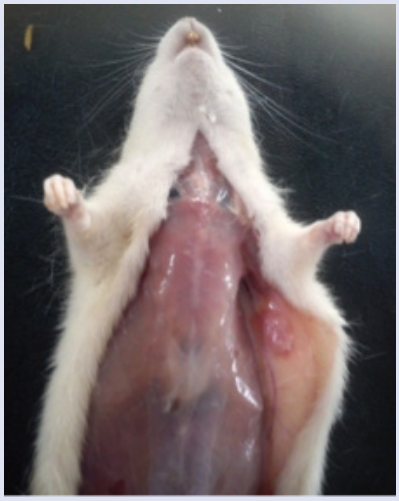

Figure 4: Intact single tumor of SFNFCUD treated rat.

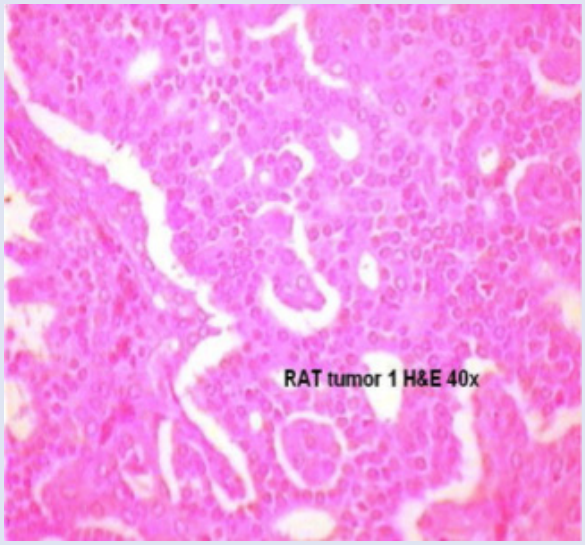

Figure 5: Intraductal papilloma with focal ductal carcinoma *in situ* (DCIS) with massive stromal and inflammatory response in MNU treated rats.

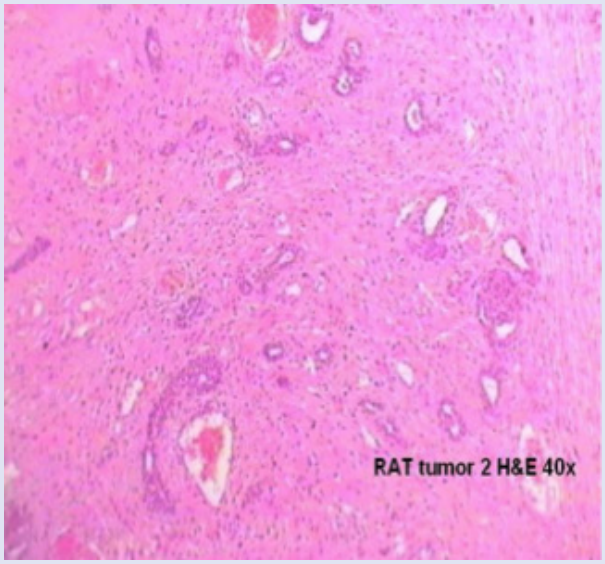

Figure 6: Fibroadenoma with moderate stromal and epithelial response in SFNF treated rats.

DISCUSSION

Nutraceuticals are natural bioactive chemical compounds that have value in health promoting, disease preventing value or semi-medicinal properties. It may range from isolated nutrients, herbal products, dietary supplements, and diets to genetically engineered 'custom' foods and processed products such as cereals, soups, and beverages.[11,12] In recent years, there has been an increased interest in the concept of whole-food synergy. Further, fortification refers to the practice of deliberately increasing the content of essential components. With this ideology, in our study, we developed four types of nutraceutical formulations, i.e. NF, SFNF, NFCUD and SFNFCUD.

All the components used in the preparation of nutraceutical formulations were of natural origin and are frequently contaminated by bacteria and fungi. Further, the presence of microbial contaminant in nonsterile pharmaceutical products can reduce or even inactivate the therapeutic activity of the products and has the potential to adversely affect patients taking the medicines.[13] WHO limits of microbial contamination in herbal materials are 10^5 cfu/g of sample in case of bacterial load; 10^3 cfu/g of sample in case of fungal load.[14] In our investigation, we found that both bacterial and fungal loads were lower than the acceptable range suggesting that all the four nutraceutical formulations are safe to use.

The health effect of polyphenols depends on the amount polyphenol concentration and their bioavailability.[15] In our study, we found that the total polyphenolic content was greatly enhanced in self-fortified nutraceutical formulation (SFNF) as compared to nutraceutical formulation (NF) suggesting that the self-fortification process can enhance the active constituents concentration.

MNU targets various organs in a variety of animal species. MNU-induced

carcinogenesis can be used as organ-specific animal models for human cancer and MNU has been most extensively utilized for the induction of mammary cancer in rats.[16] In this study, self-fortified nutraceutical formulation (SFNF) had shown better inhibitory action on MNU-induced mammary tumors as compared to plain nutraceutical formulation (NF). The possible reason for this greater anticancer potential of SFNF is that may be self-fortification increased the concentration of constituents that are responsible for anticancer activity as seen with increased total phenolic content after fortification.

Cow urine is known for its anticancer properties and has been patented for its bioenhancing property. Cow urine distillate is more effective as a bioenhancer than cow urine, and increases the effectiveness of antimicrobial, antifungal, and anticancer drugs.[17,18] Hence, NFCUD and SFNFCUD were prepared by fortifying NF and SFNF with cow urine distillate. In this study, nutraceutical formulation fortified with cow urine distillate; NFCUD and SFNFCUD showed better inhibitory action on MNU-induced mammary cancer as compared to NF and SFNF respectively suggesting the bioenhancing or potentiation of anticancer potential of NF and SFNF by cow urine distillate. However, the bioenhancing or potentiation effect was quite better with NFCUD than SFNFCUD, but insignificant in both the cases when compared without cow urine distillate. This synergetic or bioenhancing effect of cow urine distillate may be either due to the anticancer potential of cow urine distillate itself or its ability of enhancing the transport of constituents the across the gut wall.

CONCLUSION

In conclusion, the prepared nutraceutical formulations were stable, safe to use, and administration of nutraceutical formulations for 24 weeks inhibited the mammary carcinogenesis in MNU-treated Sprague Dawley rats. Further, the process of fortification is novel and enhanced the anticancer potential of the formulations, and so it can be useful.

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Conflicts of interest

There are no conflicts of interest.

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